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Issue: *Aging, Cancer, and Age-Related Diseases***Hyperbaric oxygen treatment induces antioxidant gene expression**Cassandra A. Godman,¹ Rashmi Joshi,¹ Charles Giardina,¹ George Perdrizet,² and Lawrence E. Hightower¹¹Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut. ²Department of Surgery, Morristown Memorial Hospital, Morristown, New Jersey

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Although the underlying molecular causes of aging are not entirely clear, hormetic agents like exercise, heat, and calorie restriction may generate a mild pro-oxidant stress that induces cell protective responses to promote healthy aging. As an individual ages, many cellular and physiological processes decline, including wound healing and reparative angiogenesis. This is particularly critical in patients with chronic non-healing wounds who tend to be older. We are interested in the potential beneficial effects of hyperbaric oxygen as a mild hormetic stress on human microvascular endothelial cells. We analyzed global gene expression changes in human endothelial cells following a hyperbaric exposure comparable to a clinical treatment. Our analysis revealed an upregulation of antioxidant, cytoprotective, and immediate early genes. This increase coincided with an increased resistance to a lethal oxidative stress. Our data indicate that hyperbaric oxygen can induce protection against oxidative insults in endothelial cells and may provide an easily administered hormetic treatment to help promote healthy aging.

Key words: hyperbaric oxygen therapy; oxidative stress; antioxidant genes; aging; Nrf2 transcription factor; endothelial cells

Introduction

Aging is a complex biological process that has been attributed to several mechanisms. Currently there is only a limited and somewhat superficial knowledge of the molecular mechanisms involved in aging and longevity. The interplay between cellular stressors such as reactive oxygen species (ROS) and protective antioxidant responses is generally understood to be an important factor for determining lifespan. Factors that act to increase resistance to stress, such as antioxidant enzymes and molecules, were long suspected to have anti-aging benefits.¹ In fact, studies in model organisms like *C. elegans*, *D. melanogaster*, and *M. musculus* have linked a number of genetic loci with an enhanced stress resistance and likewise an increased lifespan.¹

Rattan and colleagues have attributed the process of aging to a progressive loss of cellular function.² This change, which is defined by in-

creased molecular heterogeneity, leads to greater susceptibility to disease and ultimately death. The concept of molecular heterogeneity has been frequently invoked to explain individual variability in the aging process. This variability is described as the interaction among genetic traits, the environment, and random events, such as spontaneous genetic mutation. Stress-induced hormesis may be one effective way to reduce this accumulation of molecular damage, thus promoting healthy aging.³

The phenomenon known as hormesis is a process that results in a functional improvement of cellular stress resistance, survival, and longevity in response to sub-lethal levels of stress. It has been proposed that hormesis can promote healthy aging. One example of unhealthy aging is the decrease of angiogenesis that occurs in aged individuals, which may be a major factor in slower wound healing.² It has been shown that a sub-lethal heat

stress to vascular endothelial cells improves the formation of vascular tubules *in vitro*, which should accelerate angiogenesis and improve wound-healing capabilities.⁴

The most widely employed hormetic stresses include exercise, calorie restriction, heat stress, and pro-oxidants. Exercise requires an increase in ATP production, which leads to an increased metabolism, resulting in an increased production of ROS. Elevated ROS induces endogenous defense mechanisms such as oxidative damage repair enzymes and antioxidants. The mild oxidative stress generated through regular exercise has been associated with a decreased incidence of ROS-related diseases, such as heart disease, type II diabetes, and rheumatic arthritis.⁵ Alternatively, calorie restriction serves to slow metabolism and reduce production of ROS, again leading to reduction in the appearance of ROS-related disease.⁶ The first non-human primate study of caloric restriction without malnutrition was recently published.⁷ This study found that caloric restriction delayed the onset of age-associated diseases such as diabetes, cancer, and cardiovascular disease. Not only is there less ROS produced metabolically, but also, cells in calorie-restricted organisms are more efficient in neutralizing ROS using elevated antioxidant defenses. Transient bouts of heat stress have also been shown to increase the lifespan of *C. elegans* and other organisms.⁸ While the mechanism by which heat stress prolongs life is still under investigation, one observation our lab has made is that antioxidant genes are induced following a heat stress (unpublished). Finally, pro-oxidants have also been shown to have a hormetic effect on several organisms. Pro-oxidants such as juglone produce low levels of ROS, similar to exercise, which serves to increase endogenous protective mechanisms.⁹

Age-dependent decreases in the activity of antioxidant enzymes have been previously reported.^{10,11} Shih and Yen have suggested that changes in Nrf2 expression and MAPK regulation of the Nrf2 pathway are involved in these age-related decreases.¹¹ Likewise, Suh and colleagues have concluded that the Nrf2 signaling pathway is able to be activated upon treatment with lipoic acid in aged rats, suggesting that restoration of this protective pathway may be an effective means of slowing the aging process.¹² This key observation is important to our study because it raises the possibility that hyperbaric oxygen

therapy could reverse age-related declines in antioxidant and detoxification enzymes. Because the Nrf2 signaling pathway has the potential to activate over 200 antioxidant and cytoprotective genes in a variety of cells and tissues, it could reduce the incidence of ROS-induced, age-related diseases, effectively promoting healthy aging.

Hyperbaric oxygen (HBO) therapy has recently been promoted as an approach to slow aging. HBO treatment (HBOT) is the medical use of oxygen at higher than 1 atmosphere (atm) of pressure. The therapeutic principle behind HBO stems from increasing the partial pressure of oxygen in the tissues of the body. In addition, HBO increases the oxygen-carrying capacity of blood plasma beyond those achievable under normobaric conditions. Currently it is used to treat CO poisoning, delayed radiation injuries, decompression sickness, as well as non-healing diabetic wounds and others.¹³ Furthermore, HBO preconditioning has been shown to have hormetic effects on stress resistance and can increase the longevity of *C. elegans*.¹⁴ Although the mechanism through which HBO increases longevity is unclear, it is likely to be related to the fact that HBO can induce low levels of oxidative stressors like ROS.¹⁵ This low level of ROS could be acting to induce protective gene expression and to help cells and tissues manage various environmental and endogenous stressors more efficiently.

We wanted to address this possibility using human microvascular endothelial cells as our model system to study the effects of HBO. These cells are a direct target of HBO during wound healing, and previous research has shown that HBO can increase growth factor expression within the wound site that in turn helps stimulate angiogenesis.¹⁶ To date, studies have been limited to identifying select genes involved in the HBO mechanism. To gain a more global view of the genomic response of human microvascular endothelial cells to HBO, we performed a microarray analysis of gene expression. Using these data, we sought to identify the antioxidant and cytoprotective genes induced by HBO and whether or not their induction could protect cells against lethal oxidant stresses. In addition, we identified the cellular pathways predicted to undergo dramatic changes following HBO treatment, based upon the number of up- and down-regulated genes in each pathway.

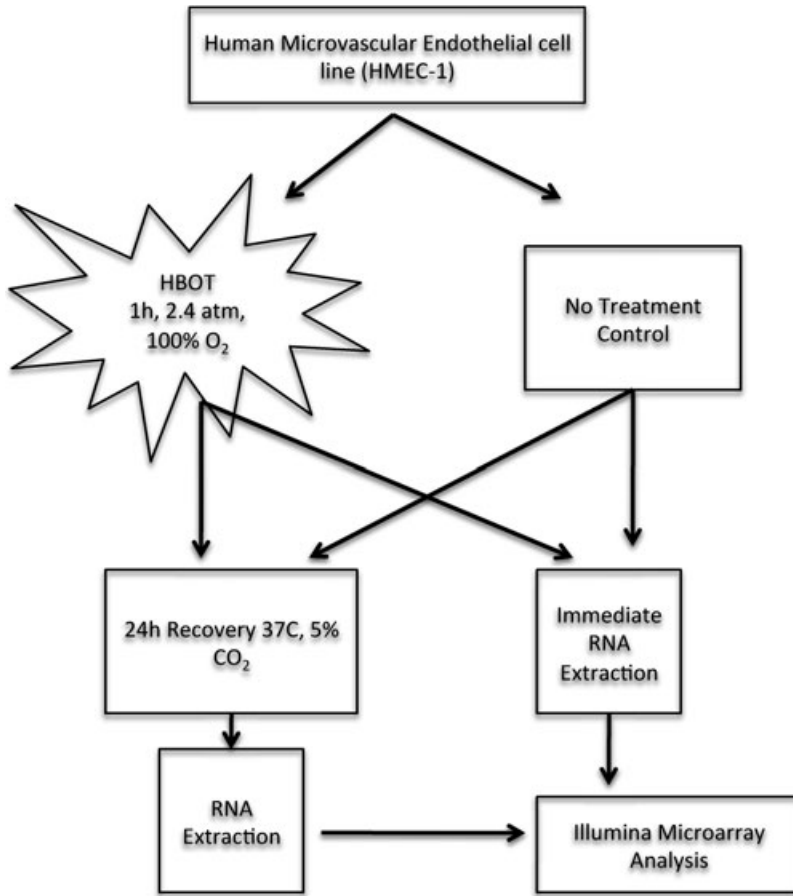


Figure 1. Experimental design. This figure illustrates the basis of our experimental design.

Experimental design

The human microvascular endothelial cell line (HMEC-1) was obtained from the Centers for Disease Control and maintained at 37°C, 5% CO₂ in MCDB131 media supplemented with 10% FBS, 1% antibiotic/antimycotic, 1 µg/ml hydrocortisone, and 10 ng/ml human epidermal growth factor. Cells were grown to 80–90% confluency and treated with our standard HBO protocol (1 h, 2.4 atm, 100% O₂) in CO₂-independent media. Control cells received a media change only. mRNA was either extracted immediately following HBO (0 h) or following a 24 h recovery in normal conditions. Resulting RNA was quantified and run on an Illumina microarray (Fig. 1). Intensity values were subjected to normalization and statistical analysis. Results for selected genes were validated using quantitative PCR (qPCR). For the viability assay, cells were exposed

to varying concentrations of *t*-butyl hydroperoxide after HBOT and allowed to recover for 16 h in normal culture conditions before being subjected to the MTT assay (Promega, Madison, WI, USA).

Results

Figure 2 illustrates selected results from the microarray. Intensity values from the array were normalized and analyzed using Limma, SAM, or SPH statistical packages to determine differentially expressed genes. Log₂ fold-changes for selected cytoprotective genes are shown (left panel). Genes uncovered in this experiment include the 70-kilodalton heat shock protein (HSPA1A), heme oxygenase 1 (HMOX1), and metallothionein 1X (MT1X), which collectively can provide protection from metabolic, proteotoxic, and oxidative forms of stress. Analysis also implicated ERK/MAPK signaling, including the

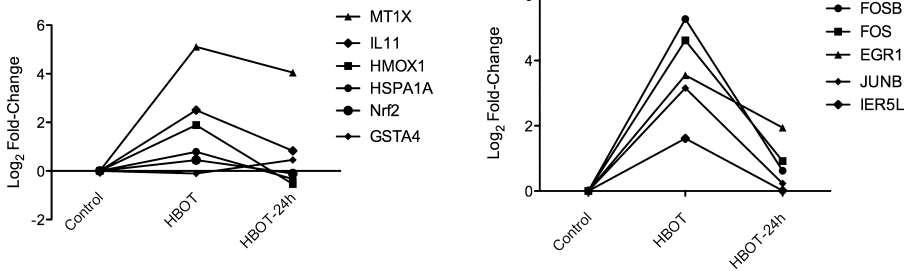


Figure 2. Activation of cytoprotective genes following HBOT. These graphs show log₂ fold-changes of select antioxidant (*left panel*), cytoprotective (*left panel*), and immediate early (*right panel*) genes among the different treatments and recovery periods.

activation of a number of immediate early genes that can potentially influence apoptotic signaling (*right panel*). Further analysis is underway to determine whether these changes affect cell survival.

Figure 3 shows the results of qPCR following HBOT and various recovery periods. HMEC-1 cells were subjected to HBOT and allowed to recover for indicated times before RNA extraction and qPCR. Relative expression for four antioxidant genes is shown (**P* < 0.05 when compared to control levels). All genes have a peak activation point following HBOT, and most center on the 4 h time point.

Figure 4 illustrates that these changes in gene expression translate to protection against a lethal oxidative stress. HMEC-1 cells were plated and allowed to attach for 48 h, at which time they were subjected to one round of HBO treatment (100% O₂, 2.4 atm, 1 h) and allowed to recover in normal culture conditions for 16 h. Cells were treated with *t*-butyl hydroperoxide (*t*-butyl OOH) at varying concentrations for 6 h and then analyzed for cell viability using an MTS assay. Cell viability in the HBO-treated cultures was significantly increased under all the conditions tested (*P* < 0.05). HBO preconditioning increased the number of viable cells in the cultures treated with HBO, indicating both a general stimulatory and a protective effect of HBO on endothelial cell viability. These findings raise the possibility that the protection afforded by HBO may arise from the coordinated expression of antioxidant and cytoprotective genes. Major targets for protection by proteins encoded by both groups of genes are likely to be proteins sensitive to oxidative damage, denaturation, and aggregation.

Discussion

Our results indicate that HBOT can change gene expression in a number of cellular pathways, including the Nrf2, Integrin, and ERK/MAPK pathways. These pathways contain a number of genes that are critical in cellular defense. The functional significance of these gene expression changes is supported by our finding that HBO-treated cells are protected against an otherwise lethal oxidative stress. These findings support the potential benefit of HBO in a number of clinical applications. For example, a pre-conditioning HBO treatment may be an attractive option for patients about to undergo particularly stressful operations, including open-heart surgeries. A recent study has demonstrated the ability of HBO pretreatment to protect humans from acute ischemia during coronary artery bypass grafting procedures employing cardiopulmonary bypass.^{17,18} More generally, HBO could be a viable treatment option to slow aging because of its ability to increase endogenous antioxidant enzymes that suppress ROS-induced cellular damage. We have shown that after just one round of HBO, expression of these protective genes increases significantly, and this single treatment protects cells against a ROS insult. Overall, HBO is a relatively low-risk procedure that could be effectively applied as a broader preventative regimen to reduce the effects of aging. Ultimately, we would like to link these cell culture studies with human studies to demonstrate that HBO-induced gene expression changes also occur in patients where they provide a similar level of protection.

Analysis of the gene array data also show that HBO can stimulate proliferation of HMEC-1 cells

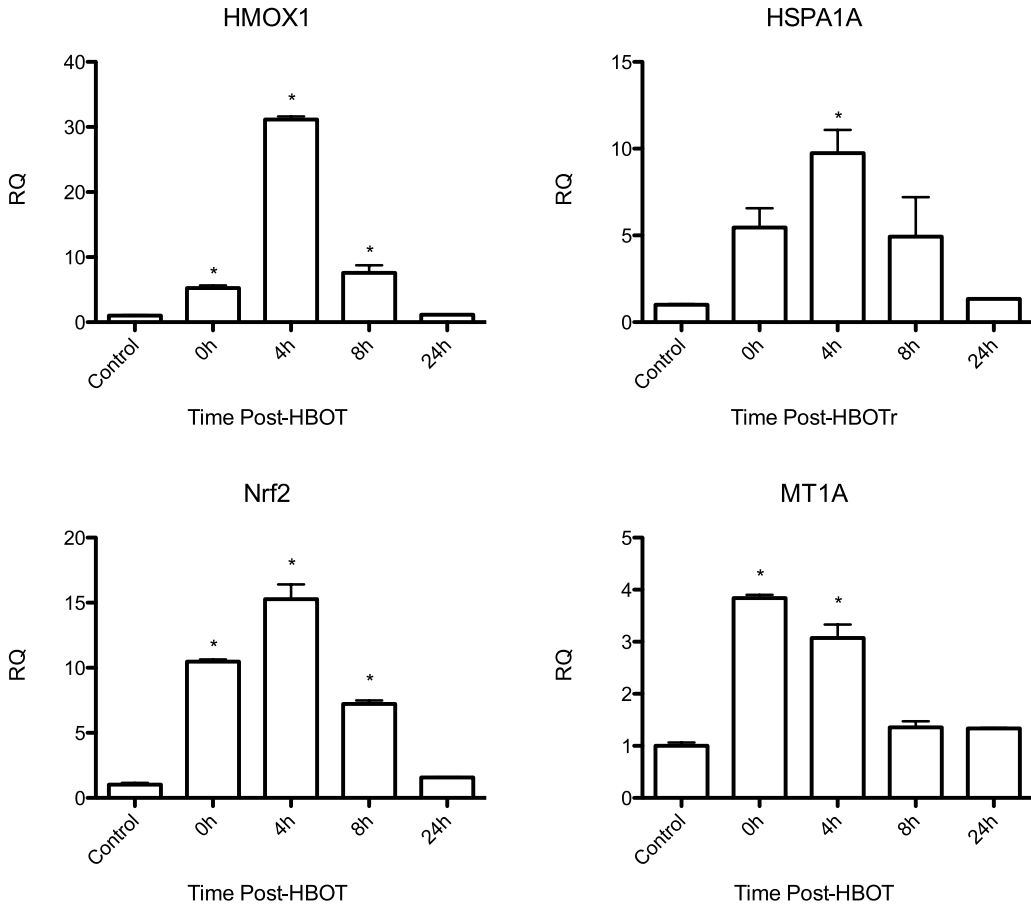


Figure 3. Validation of gene expression changes at various time points following HBOT. qPCR was used to confirm some gene expression changes seen on the microarray. In addition to confirmation of changes, a time-course study was carried out to determine the optimal activation time following HBOT. Graphs show relative expression compared to control, untreated cells.

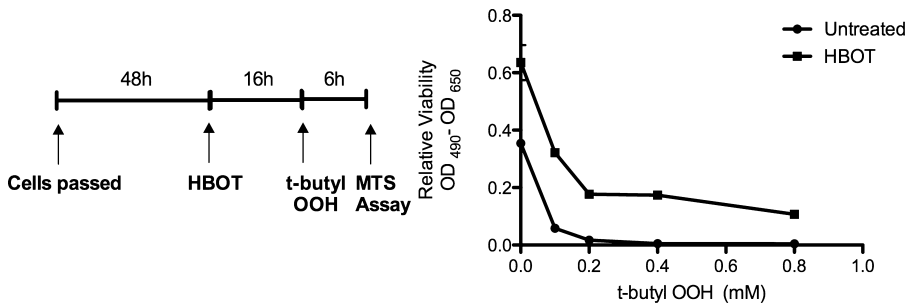


Figure 4. HMEC-1 cells treated with HBOT were resistant to oxidative stress. *Left panel* demonstrates the experimental design. The MTS assay revealed an enhancement of cell growth following HBOT. Cells also treated with *t*-butyl OOH survived higher concentrations of the oxidant compared to untreated cells (*right panel*).

through the activation of growth-regulatory genes. Endothelial cell proliferation is an important component of angiogenesis and wound healing, both of which decline with age. Our results also support the idea that HBO is a hormetic agent that activates antioxidant and cytoprotective genes to later protect against a lethal insult. Of the hormetic agents that have been proposed for human use as anti-aging treatments, HBO has the advantages of minimal side effects and a track record of safe FDA-approved use for a variety of indications. To be effective, this treatment likely depends upon reversal of age-related declines in Nrf2 signaling. If studies in rats like those described above are applicable to humans, HBOT becomes an appealing anti-aging therapeutic option for the human population. However, additional research is required to establish the beneficial effects of HBO in humans and to devise specific clinical protocols that maximize protective responses of this hormetic treatment without risking potential damaging effects of excessive oxygen exposure.

Conflicts of interest

The authors declare no conflicts of interest.

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